Growth Factor Expression With Different Wound Treatments After Laser Resurfacing

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Background: *Many studies have demonstrated that the specific method of wound dressing used may affect the healing process. However, the effect of the method of wound dressings on the expression of growth factors is not well documented.*

Objective: The aim of this study was to evaluate the effects of different methods of treatment on the healing process and the expression of growth factors (epidermal growth factor, basic fibroblast growth factor, transforming growth factor–β_2 [TGF-β_2], platelet-derived growth factor–A, and platelet-derived growth factor–B) by histologic study, immunohistochemistry, and reverse transcription–polymerase chain reaction.

Methods: *In this study, we produced wounds with a CO_2 laser on the backs of rats and used 4 different methods of wound treatment: occlusive dressing material, petrolatum ointment, β-sitosterol ointment, and exposure to air (untreated) as a control. Five-millimeter biopsy specimens were obtained 1, 3, 5, 7, and 10 days after surgery for histologic evaluation and expression of growth factors from four different dressing sites.*

Results: *By microscopic examination, there was an acceleration of wound healing in the occlusive dressing wounds, as well as a lesser improvement in healing times with the petrolatum and β-sitosterol–treated wounds, compared with the air-exposed control subjects. With immunohistochemistry, we observed that the tissue expression of TGF-β_2 remained at a clearly lower level during the entire duration of wound healing in the occlusive dressing wound compared with the other treatment wounds. With reverse transcriptase–polymerase chain reaction, however, our data did not reveal statistically significant differences among the messenger RNA levels.*

Conclusions: *Our results suggest that a decrease in the expression level of TGF-β_2 under occlusive dressings could provide an environment in which the growth of human epidermal keratinocytes and re-epithelialization is promoted.* (Aesthetic Surg J 2007;27:55–64.)

Various treatment options are available for wound healing. Standard methods include leaving the wounds exposed to air, application of ointments, and occlusion of the wound with biosynthetic dressing materials.[1,2] Many studies have demonstrated that the specific method of wound dressing used may affect the healing time.[3-6] The primary factor in the acceleration of the wound healing process is the capacity to maintain a moist environment.[7-11] In addition to moisture, various growth factors have been implicated in the wound healing process.[12-14] To the best of our knowledge, however, there is no report on the effect of the method of wound dressings on the expression of growth factors during the course of wound healing.

Therefore we attempted to investigate how the expression of growth factors is affected by the method of dressing during the healing process. In this study, we produced wounds with a CO_2 laser and used 4 different methods of wound treatment: occlusive dressing material, petrolatum ointment, β-sitosterol ointment, and exposure to air (untreated) as a control. We evaluated the effects of different methods of treatment on the healing process and the expression of growth factors by histologic study, immuno-

histochemistry, and reverse transcription–polymerase chain reaction (RT-PCR).

Materials and Methods

Animals

Sprague-Dawley rats were housed individually in a controlled facility operated by the Yale Animal Resources Center. Animals received food and water as desired. All animal procedures were approved by the Yale Animal Care and Use Committee.

Preparation of wound tissue

Nine Sprague-Dawley rats (weight 250-300 g) were sedated with an intraperitoneal injection of ketamine/xylazine cocktail (ketamine 70 mg/kg, xylazine 4 mg/kg). Once adequately sedated, the paravertebral skin region was shaved with an electric hair clipper. The rat was then placed in a nosecone delivering isoflurane gas, and the skin was surgically prepared.

After sterile draping, 2 separate rectangular areas of skin resurfacing, 3 × 3 cm, were created on each of the bilateral paravertebral trunk regions with a Luxar LX 20SP Novapulse CO_2 laser (Luxar Corp., Bothell, WA). The laser was set in superpulse mode at 8W. This setting provided energy of 480 mJ with a 3-mm pattern of the NovaScan hand piece. Three passes were performed with the NovaScan 3-mm hand piece. Between successive passes, saline solution–soaked gauze was used to remove any devitalized tissues, and the field was wiped dry.

After surgery, animals were randomly divided into three groups of 3. Each treated region underwent wound treatment with one of four different methods (Figure 1): Group I: Open untreated (air exposed); petrolatum ointment (Petroleum Jelly: Vi-Jon Laboratories, St. Louis, MO). Group II: Open untreated (air exposed); β-sitosterol ointment (MEBO, Beijing Guangming Chinese Medicine Institute for Burns, Wounds & Ulcers, Shantow City, China). Group III: Open untreated (air exposed); an occlusive dressing (Flexzane, Dow Hickam Pharmaceuticals, Sugar Land, TX).

Elizabethan collars (Harvard Apparatus Inc, Holliston, MA) were placed on all animals to prevent them from grooming and destroying dressings. Occlusive dressings were secured in place by 6-0 nylon stitches and changed after 24 hours if they did not stick because of the exudates. The ointments were applied once daily until complete epithelialization occurred. Five-millimeter biopsy specimens were obtained 1, 3, 5, 7, and 10 days after surgery for histologic evaluation and expression of growth factors (epidermal growth factor [EGF], basic fibroblast growth factor [bFGF], transforming growth factor–β_2 [TGF-β_2], and platelet-derived growth factor–A [PDGF-A] and –B [PDGF-B]) from 4 different

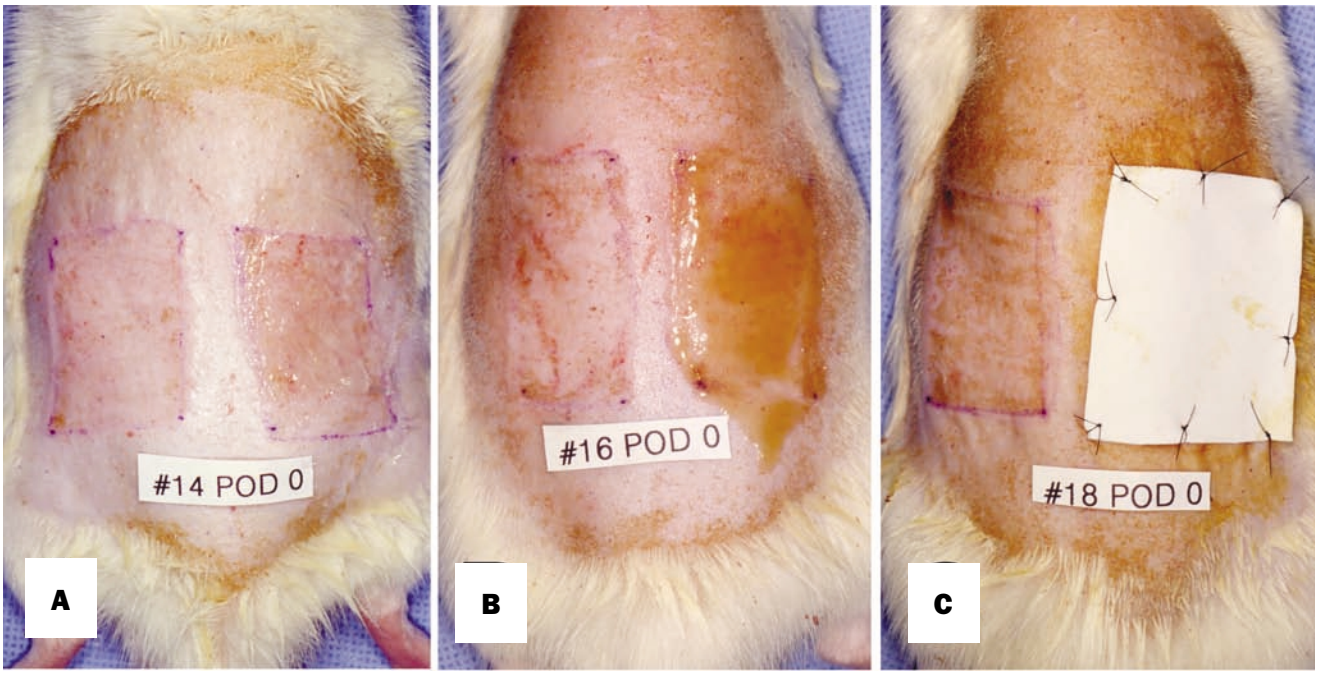

Figure 1. *The two rectangular areas underwent CO_2 laser resurfacing. Each treated region underwent wound treatment as follows:* **A,** *Group I: open untreated; petrolatum ointment;* **B,** *Group II: open untreated; β-sitosterol ointment; and* **C,** *Group III: open untreated; an occlusive dressing.*

dressing sites. As controls for immunohistochemistry and RT-PCR, one more biopsy specimen was taken outside of the laser-resurfaced area on postoperative day 1.

Histologic study

The specimens were fixed in 10% buffered neutral formalin. The tissues were then embedded in paraffin and cut into 5-µm sections for hematoxylin and eosin (H & E) staining or immunohistochemistry. A pathologist evaluated the specimens for scale crust formation, percent epithelialization, inflammation, and final histologic appearance via light microscopy.

Immunohistochemistry

To observe the differences in the expression of growth factors from four different methods of treatments, immunostains for EGF, bFGF, TGF-β_2, PDGF-A, and PDGF-B were performed on skin specimens 1, 3, 5, 7, 10 days after CO_2 laser resurfacing, as well as nonwounded control skin.

Rabbit anti-EGF antibody was obtained from Sigma Chemical Company (St. Louis, MO). Polyclonal rabbit anti–TGF-β_2 antibody, polyclonal rabbit anti-bFGF antibody, polyclonal rabbit anti–PDGF-A antibody and polyclonal rabbit anti–PDGF-B antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After sectioning, the tissues were deparaffinized and rehydrated, endogenous peroxidase was blocked by quenching with hydrogen peroxide and methanol. After preblocking with Dako blocking agent (Dako Corporation, Carpinteria, CA) for 5 minutes, primary antibody was applied for 20 minutes at room temperature. The Dako LSAB2 peroxidase, DAB secondary detection system, was used with DAB as a chromogen resulting in a brown stain. Last, the sections were washed with distilled water and counterstained with Gill III hematoxylin. Immunostaining observations revealed positive areas of growth factors (Figure 2).

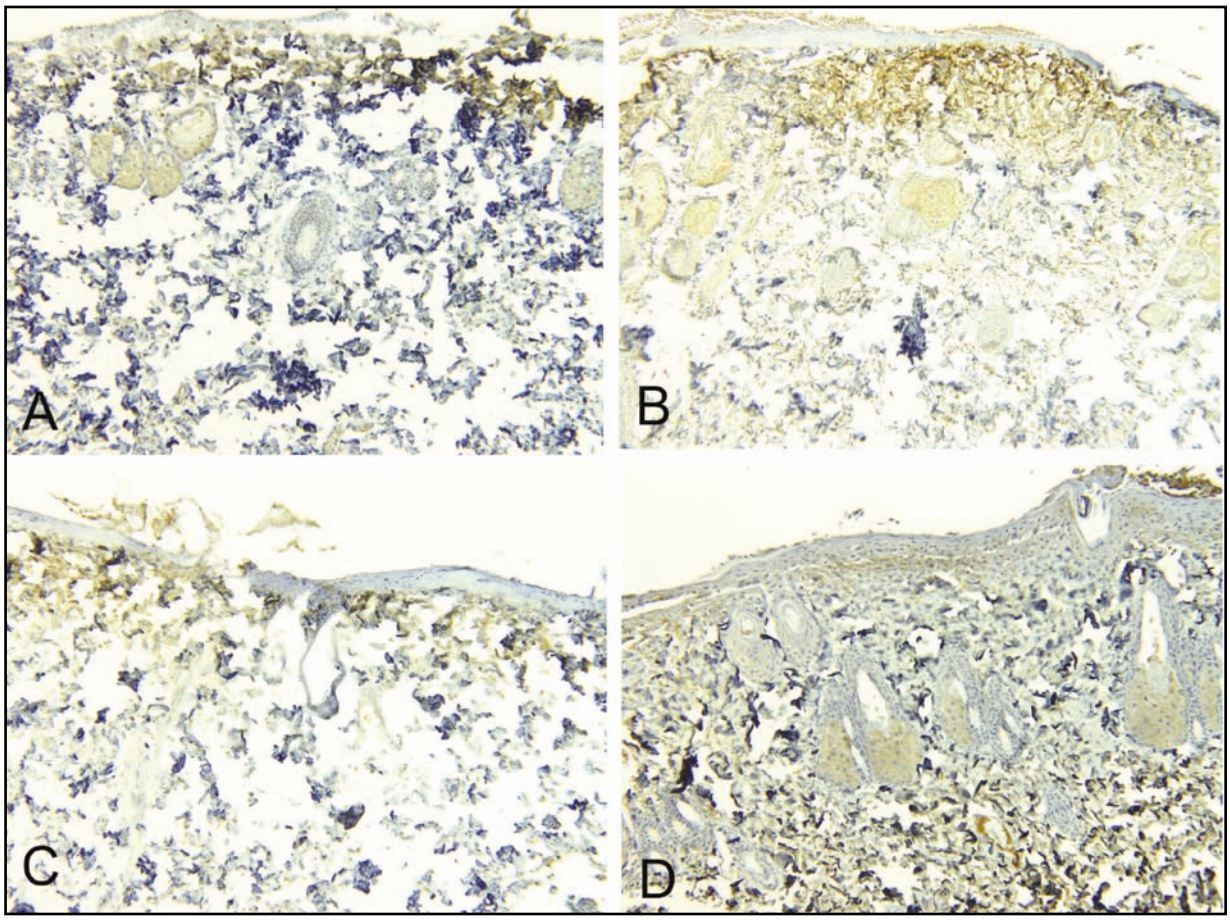

Figure 2. *Immunohistochemistry shows positive staining of TGF-β_2 (brown color) in wounds on postoperative day 1.* **A,** *Open untreated;* **B,** *petrolatum ointment;* **C,** *β-sitosterol ointment;* **D,** *occlusive dressing. (Original magnification ×40.)*

One pathologist evaluated all of the specimens for scoring of the relative staining intensity. For each of the slides the staining intensity was scored with a semiquantitative scale ranging from 0 to 3 (0 = not detectable; 1 = light staining; 2 = moderate staining; and 3 = strong staining).

mRNA analysis

mRNA levels were accessed with RT-PCR. The total cellular RNA from the specimens was extracted by use of Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Rneasy Mini Kits (Quiagen GmbH, Hilden, Germany) were then used to purify total RNA isolated. In RT-PCR assays, 1 μg of RNA was reverse transcribed to cDNA, and gene-specific primers were used to amplify selected regions of each target moiety (Table 1). The whole reaction was performed in a 25-μL volume with an Access RT-PCR kit purchased from Promega (Madison, WI) according to the manufacturer's instructions. All primers were synthesized at the Yale Oligonucleotide Synthesis Laboratory.

To verify that equal amounts of undegraded RNA were added in each RT-PCR reaction, β-actin was used as an internal standard. Amplified PCR products were fractionated on 1.2% agarose gels and stained with homidium bromide. The homidium bromide bands were visualized under ultraviolet light, photographed, and scanned into a computer. Luminescence of homidium bromide staining for the PCR products was determined with the Alpha Imager 2000 (Alpha Innotech. Corporation, San Leandro, CA). The ratio between determined value for the cytokines and β-actin was calculated to compare the samples of skin specimens obtained.

Statistical analysis

A Mann-Whitney *U* test was performed to detect statistically significant differences in the mRNA expression levels of each of the growth factors among the differently treated groups on days 1, 3, 5, 7, and 10 by RT-PCR after wound production. Statistical significance was set at $P < .05$.

Results

Histologic study

There were differences among the differently treated wounds in the amount of cellular infiltrate, presence of scale crust, percent of epithelialization, and degree of inflammation.

On day 1 after wound production, the epidermal layer was absent in all the sites. Mild focal inflammation, immediately beneath the surface, was observed in the petrolatum- and β-sitosterol–treated wounds, with less inflammation in the air-exposed and occlusive dressing wounds.

On day 3 after wound production, the lasered site was covered with a thick scale crust in the air-exposed wounds. The newly forming epithelium tunneled under this devitalized area, and one third of the treated site was

Table 1. Primer sequences and RT-PCR conditions

Gene	s/as	Primer sequences (5′ to 3′)	Anneal. Tm.	Cycle	Product size (bp)	Genbank accession number
EGF	s	CTTGCAGAATCACGGCTGTA	60°C	30	418	NM_012842
	as	GCATGTGTCGTATGTCCTGG				
bFGF	s	TATGAAGGAAGATGGACGGC	60°C	30	167	M22427
	as	CCGTTTTGGATCCGAGTTTA				
TGF-β_2	s	GCAGAGTTCAGGGTCTTTCG	60°C	30	349	NM_031131
	as	CACCACTGGCATATGTGGAG				
PDGF-A	s	CAAGACCAGGACGGTCATTT	60°C	30	122	D10106
	as	CCAGCTCATCTCACCTCACA				
PDGF-B	s	GAGTGCAAGACGCGTACAGA	60°C	30	136	L40991
	as	ACTGCACATTGCGGTTATTG				
β-Actin	s	GTGGGCCGCTCTAGGCACCA	60°C	25	241	X03672
	as	TGGCCTTAGGGTTCAGGGGG				

Anneal. Tm., annealing temperature; as, antisense; s, sense.

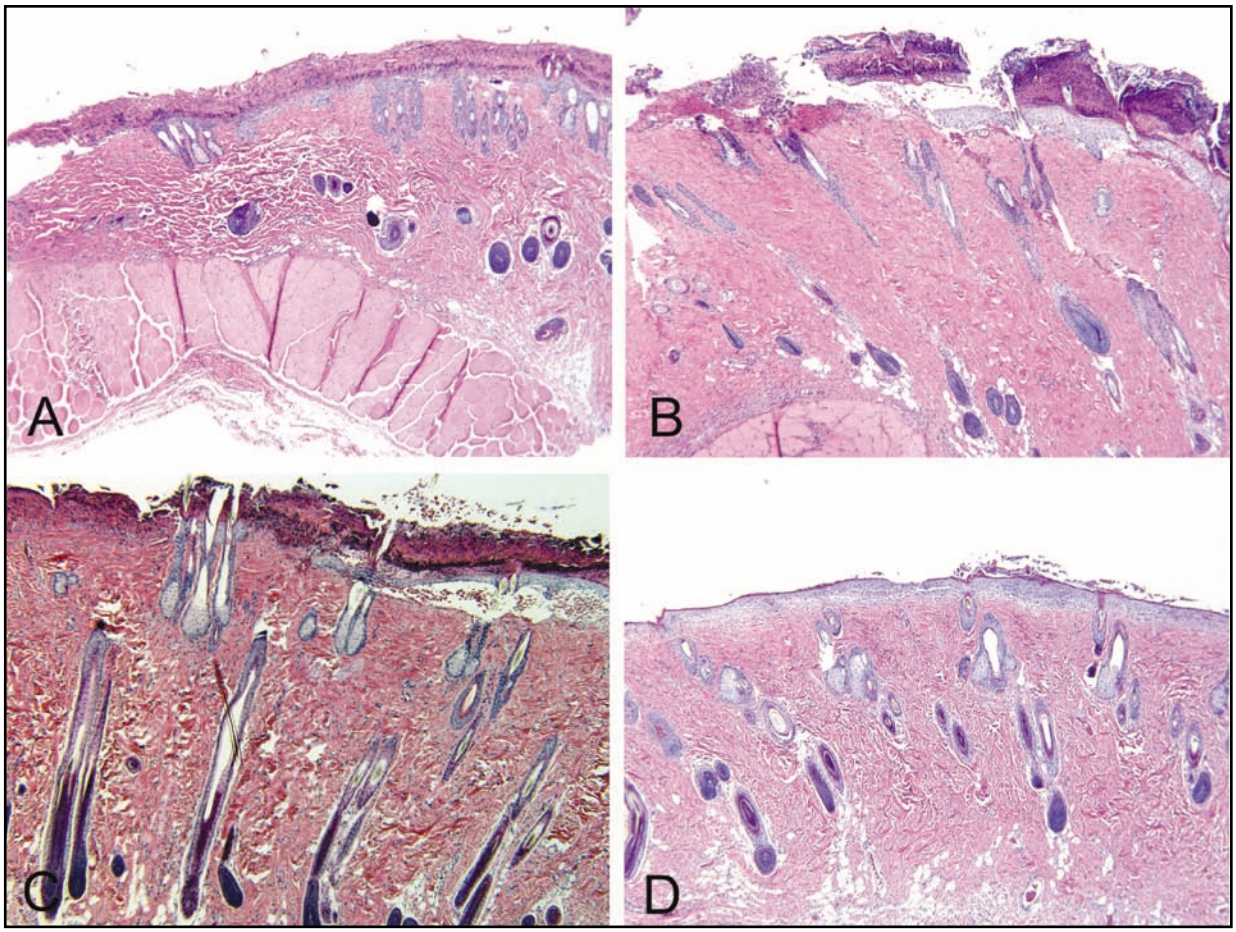

Figure 3. *Histologic study of skin samples on postoperative day 3.* **A,** *Open untreated;* **B,** *petrolatum ointment;* **C,** β*-sitosterol ointment;* **D,** *occlusive dressing. (H & E staining; original magnification ×40.)*

epithelialized. In the petrolatum- and β-sitosterol–treated wounds, however, a thicker scale crust covered the epithelialized and non-epithelialized areas. The epithelialization rate was 70% in the petrolatum-treated wounds and 50% in the β-sitosterol–treated wounds. In the occlusive dressing wounds, a focal overlying thin scale crust was observed, and there was almost complete reepithelialization on day 3 (Figure 3).

Complete reepithelialization occurred by day 5 in the petrolatum- and β-sitosterol–treated wounds, and no microscopic difference was observed between them. On day 7 after wound production, all the treatment sites, as well as the air-exposed control subjects, were 100% epithelialized (Figure 4).

Immunohistochemistry

Among five different growth factors, EGF, bFGF, PDGF-A, and PDGF-B demonstrated similar patterns to each other, even when the dressing methods were different. They were increased immediately after resurfacing and demonstrated the strongest intensity from day 1 to day 3. After their peak levels, they decreased and then returned to normalize gradually over the duration of the experiment. The strongest intensity of immunostaining in the wound occurred on day 1 for EGF, bFGF, and PDGF-B and on day 3 for PDGF-A.

The expression of TGF-β_2 exhibited a similar pattern to that observed with the other growth factors in the air-exposed and ointment-treated wounds. On the other hand, immunostaining of TGF-β_2 was different in the occlusive dressing wounds. In these sites, the expression of TGF-β_2 decreased from the first day after wound production and remained at a lower level compared with those in air-exposed and ointment-treated wounds throughout the duration of this study. The lowest intensity of immunostaining occurred on day 5 (Figure 5).

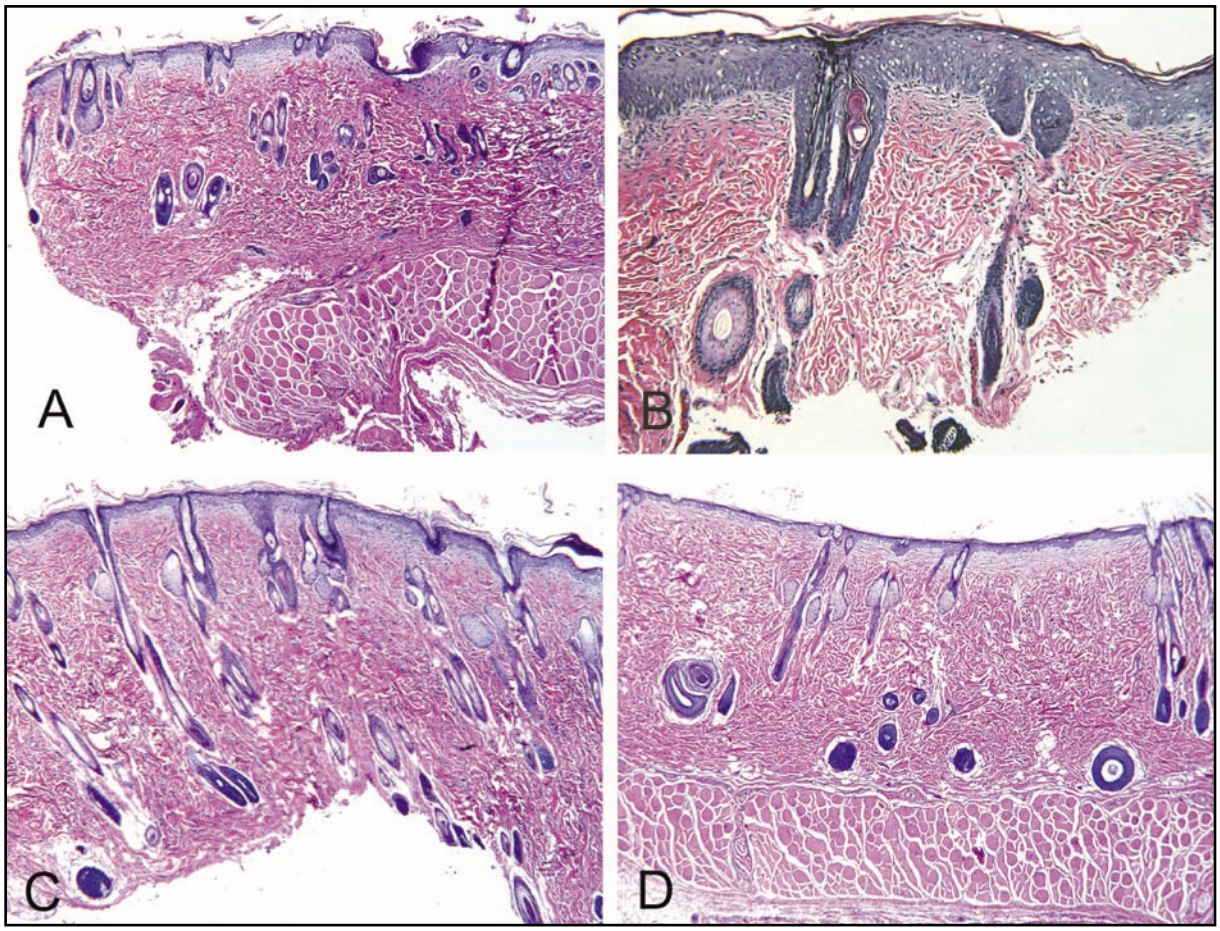

Figure 4. *Histology of skin samples on postoperative day 7.* **A,** *Open untreated;* **B,** *petrolatum ointment;* **C,** β-*sitosterol ointment;* **D,** *occlusive dressing. (H & E staining; original magnification ×40.)*

mRNA assay

RT-PCR was performed on mRNAs that were isolated from each of the control subjects and samples with different methods of treatment on days 1, 3, 5, 7, 10 after laser resurfacing (Figure 6). Densitometry values for EGF, bFGF, TGF-β_2, PDGF-A, and PDGF-B were corrected to a β-actin expression at each time point, normalized by setting the control value to 1, and depicted graphically as the mean ±SD.

Statistically, there was no significant ($P > .05$) difference in the mRNA expression levels of each of the growth factors among the differently treated wounds on days 1, 3, 5, 7, and 10 by RT-PCR after wound production. When we analyzed the data, we did not observe any consistency or pattern of the expression of the growth factors among the differently treated sites on the days after wound production (Table 2).

Discussion

In this study, we examined the histologic changes of wound healing and the expression of five different growth factors at differently treated wound sites in rats over a 10-day period.

At first, by microscopic examination, we confirmed that healing time was influenced by the dressing materials used, as demonstrated in previous studies.[15,16] There was an acceleration of wound healing in the occlusive dressing wounds, as well as a lesser improvement in healing times with the petrolatum- and β-sitosterol–treated wounds, compared with the air-exposed sites.

The mechanisms that underlie the promotion of reepithelialization by an occlusive dressing are still not understood completely. Eaglstein[17] discussed 5 possibilities: easier migration of epithelial cells, increased partial pressure of oxygen, increased local concentration of

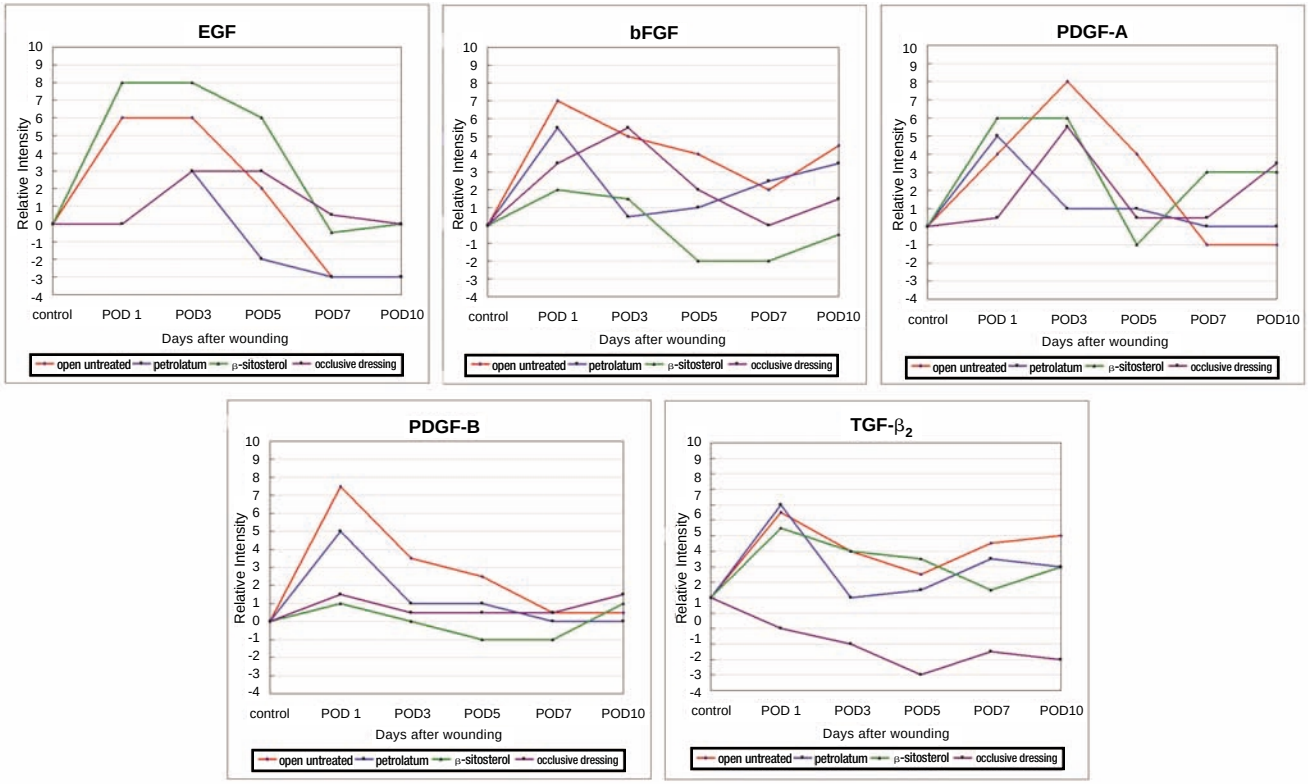

Figure 5. *Pattern of immunostaining for EGF, bFGF, PDGF-A, PDGF-B, and TGF-β_2 with different wound treatments after CO_2 laser resurfacing.*

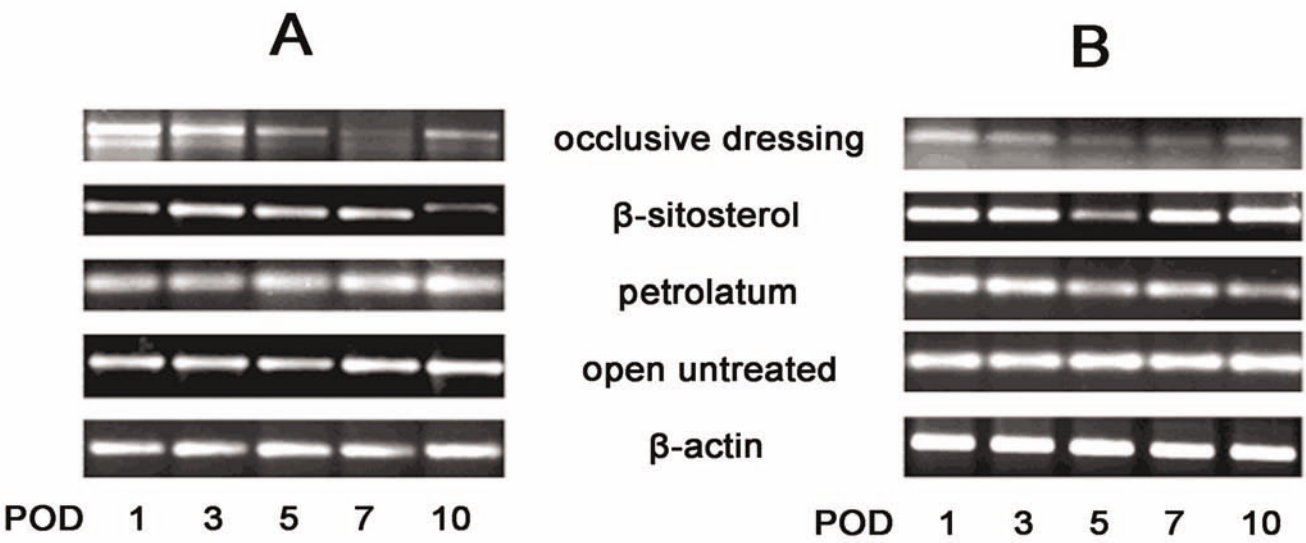

Figure 6. *RT-PCR of TGF-β_2 and PDGF-B with different wound treatments after CO_2 laser resurfacing.* **A,** *TGF-β_2;* **B,** *PDGF-B.*

Table 2. Expression of mRNA for growth factors 1, 3, 5, 7, 10 days after wounding

Group I

Growth factor	Rat No.	Open untreated						Petrolatum ointment				
		Control	Day 1	Day 3	Day 5	Day 7	Day 10	Day 1	Day 3	Day 5	Day 7	Day 10
EGF	1	1.56	1.89	1.97	2.20	2.89	1.20	2.10	2.23	3.40	1.73	1.39
	2	0.13	0.12	0.88	0.38	0.12	0.14	0.22	0.28	0.15	0.42	0.05
	3	0.16	0.12	0.17	0.61	0.13	0.73	1.02	0.95	0.25	0.21	0.92
bFGF	1	1.20	1.67	1.29	1.65	1.88	0.91	1.54	1.26	2.61	1.11	1.19
	2	0.61	0.65	0.45	0.76	0.40	0.60	1.24	0.87	1.07	1.38	0.81
	3	0.97	0.54	0.83	0.64	0.43	0.88	0.80	1.09	1.03	0.73	1.30
TGF-β_2	1	1.85	2.73	1.49	2.17	1.41	1.41	1.23	1.75	2.10	1.45	1.46
	2	0.63	0.29	0.20	0.56	0.10	0.26	0.13	0.77	0.72	0.51	1.04
	3	0.16	0.07	0.09	0.12	0.15	1.17	0.31	0.47	0.95	0.42	1.38
PDGF-A	1	2.21	3.64	2.34	2.40	3.14	1.68	2.10	2.14	4.35	1.32	2.13
	2	1.54	0.53	0.95	1.84	1.33	1.17	1.03	1.31	1.43	2.00	1.27
	3	0.00	0.00	1.09	0.55	0.11	1.34	1.13	1.11	1.51	1.12	1.56
PDGF-B	1	2.73	2.42	2.27	2.80	3.14	1.41	2.00	2.39	4.67	1.61	1.05
	2	0.59	0.15	1.26	1.07	0.63	0.74	0.63	0.83	0.64	0.78	0.92
	3	0.87	0.46	0.98	0.76	0.70	1.17	1.39	1.07	0.77	0.60	1.07

Group II

Growth factor	Rat No.	Open untreated						β-sitosterol ointment				
		Control	Day 1	Day 3	Day 5	Day 7	Day 10	Day 1	Day 3	Day 5	Day 7	Day 10
EGF	4	0.17	0.31	0.51	0.08	0.24	0.47	0.27	0.28	0.16	0.22	0.43
	5	0.41	0.06	0.48	0.53	0.46	0.48	0.24	0.25	0.77	0.25	0.38
	6	1.11	0.84	1.58	0.88	3.73	2.42	1.15	6.84	2.12	3.57	1.40
bFGF	4	0.62	0.51	0.52	0.53	0.58	0.51	0.62	0.60	0.73	0.68	0.61
	5	0.36	0.21	0.45	0.21	0.49	0.54	0.62	0.43	0.20	0.43	0.69
	6	1.90	3.34	1.79	1.80	2.73	3.25	2.32	3.25	1.39	2.50	2.40
TGF-β_2	4	0.13	0.10	0.11	0.17	0.06	0.13	0.12	0.11	0.35	0.24	0.39
	5	1.02	0.11	0.44	0.17	1.23	1.32	0.11	0.60	0.15	1.03	0.83
	6	2.86	2.72	2.47	2.67	4.27	5.13	1.29	2.17	2.23	2.58	1.80
PDGF-A	4	0.38	0.39	0.56	0.54	0.37	0.63	0.62	0.50	1.15	0.68	0.88
	5	1.11	0.13	1.29	0.64	0.99	1.46	1.51	0.55	1.61	1.43	1.76
	6	1.39	2.17	2.18	2.50	3.47	3.46	2.61	4.75	3.58	2.22	3.20
PDGF-B	4	0.32	0.55	0.36	0.37	0.65	0.37	0.79	0.72	0.69	0.61	0.48
	5	1.68	0.23	0.74	1.57	1.84	1.97	2.02	2.00	0.98	1.56	1.69
	6	2.54	3.84	1.32	3.33	5.96	4.08	4.08	9.59	6.58	9.43	7.40

Group III

Growth factor	Rat No.	Open untreated						Occlusive dressing				
		Control	Day 1	Day 3	Day 5	Day 7	Day 10	Day 1	Day 3	Day 5	Day 7	Day 10
EGF	7	0.61	1.16	2.27	0.85	0.71	0.70	1.13	0.51	0.72	0.76	0.36
	8	0.60	0.93	0.78	0.07	0.58	0.57	1.10	0.40	0.81	0.45	0.31
	9	0.42	0.55	0.31	0.46	0.40	0.14	0.58	0.23	0.50	0.71	0.33
bFGF	7	1.55	3.24	2.02	1.37	1.98	1.34	1.67	1.62	1.36	1.72	1.23
	8	0.33	1.03	1.18	0.86	1.07	0.78	1.56	0.98	0.92	0.86	1.15
	9	1.08	0.58	0.55	0.86	0.88	0.49	0.46	0.70	0.40	0.70	0.25
TGF-β_2	7	1.12	1.11	1.44	0.87	0.85	0.79	0.99	1.03	1.30	1.56	0.77
	8	0.75	0.50	0.45	0.11	0.43	0.55	1.08	1.02	1.26	1.34	0.82
	9	0.72	0.45	0.74	0.85	1.21	0.45	0.83	0.99	0.92	0.84	0.46
PDGF-A	7	3.42	3.31	3.19	3.06	2.89	1.51	2.11	2.02	1.71	3.61	2.09
	8	1.13	0.97	1.43	1.52	1.13	1.17	1.82	1.12	1.29	2.41	1.44
	9	0.74	0.52	0.77	1.03	0.85	0.61	0.69	0.96	0.72	0.91	0.42
PDGF-B	7	2.37	2.98	4.24	1.82	1.50	1.18	1.00	0.90	1.37	2.35	1.36
	8	1.13	1.22	1.43	0.57	0.80	0.97	0.96	0.64	0.89	1.17	1.12
	9	1.40	1.37	0.68	1.04	1.24	0.84	1.09	0.92	0.76	0.90	0.50

growth factors, favorable effects of increased microbial flora, and the maintenance of an electrical potential between the wounded skin and the surrounding normal skin. A known fact is that occlusive dressings are effective in the acceleration of wound healing by maintaining a humid wound environment.[8-11] Chvapil et al[1] hypothesized that wound dressings enhanced healing by the activation of cells that produced growth factors and other mediators of the repair process. However, they did not provide any data to confirm their hypothesis.

We assessed whether the expression of growth factors was different depending on the dressing methods used by immunohistochemistry and RT-PCR. With immunohistochemistry, we observed that there was an increase in the expression of each growth factor in all the treatment wounds after wound production, except for TGF-β_2 in occlusive dressing wounds. The tissue expression of TGF-β_2 remained at a clearly lower level during the entire duration of wound healing in the occlusive dressing wounds compared with the other treatment wounds.

TGF-β has inhibitory effects on the proliferation of normal human epidermal keratinocytes.[18,19] Normal human epidermal keratinocytes are the main component cells of the epidermis, and their growth is regulated by both positive and negative mediators.[20,21] Among the negative mediators, one of the most important growth inhibitors of normal human epidermal keratinocytes is TGF-β.[18] We suggest that a decrease in the expression level of TGF-β under occlusive dressings could provide an environment in which the growth of human epidermal keratinocytes and reepithelialization are promoted.

With RT-PCR, we could not verify the same patterns of mRNA expressions of the different growth factors, which were demonstrated in the immunohistochemistry. Although there were differences in the mRNA levels of each growth factor among the differently treated wounds, our data did not reveal statistically significant differences among the mRNA levels. This result could be explained by the individual variations of expression of the growth factors in each rat and the relatively small (n = 3, for each method of treatment) number of subjects studied. To demonstrate a statistical significance, further studies with larger numbers of samples are necessary.

Conclusion

We have evaluated the effects of different methods of wound treatment on the healing process and the expression of growth factors (EGF, bFGF, TGF-β_2, PDGF-A, and PDGF-B). With immunohistochemistry, we observed that the tissue expression of TGF-β_2 remained at a clearly lower level during the entire duration of wound healing in the occlusive dressing compared with the other treatment wounds. This result suggests that a decrease in the expression level of TGF-β_2 under occlusive dressings could provide an environment in which the growth of human epidermal keratinocytes and reepithelialization is promoted. ■

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